Prostaglandin E_2 induces fibroblast apoptosis by modulating multiple survival pathways

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ABSTRACT **Although the lipid mediator prostaglandin E_2 (PGE_2) exerts antifibrotic effects by inhibiting multiple fibroblast functions, its ability to regulate fibroblast survival is unknown. Here, we examined the effects of this prostanoid on apoptosis and apoptosis pathways in normal and fibrotic lung fibroblasts. As compared to medium alone, 24 h of treatment with PGE_2 increased apoptosis of normal lung fibroblasts in a dose-dependent manner (EC_{50}~50 nM), as measured by annexin V staining, caspase 3 activity, cleavage of poly-ADP-ribose polymerase, and single-stranded DNA levels. PGE_2 also potentiated apoptosis elicited by Fas ligand plus cycloheximide. These proapoptotic actions were dependent on signaling through the EP2/EP4 receptors and by downstream activation of both caspases 8 and 9. Silencing and gene deletion of PTEN demonstrated that the effects of PGE_2 involved decreased activity of the prosurvival molecule Akt. PGE_2 also down-regulated expression of survivin, an inhibitor of apoptosis, and increased expression of Fas. Fibroblasts from patients with pulmonary fibrosis exhibited resistance to the apoptotic effects of PGE_2. These findings show for the first time that, in contrast to its effects on many other cell types, PGE_2 promotes apoptosis in lung fibroblasts through diverse pathways. They provide another dimension by which PGE_2 may inhibit, and perhaps even reverse, fibrogenesis in patients with interstitial lung disease.—Huang, S. K., White, E. S., Wettlaufer, S. H., Grifka, H., Hogaboam, C. M., Thannickal, V. J., Horowitz, J. C., Peters-Golden, M. Prostaglandin E_2 induces fibroblast apoptosis by modulating multiple survival pathways. *FASEB J.* 23, 4317–4326 (2009). www.fasebj.org**

Key Words: PTEN · survivin · Fas · Akt · idiopathic pulmonary fibrosis

A PROPER BALANCE BETWEEN cell survival and death is critical for maintaining normal tissue homeostasis, and many diseases are characterized by a disruption in this balance (1). For example, both excess epithelial cell apoptosis (2–4) and enhanced fibroblast survival (5–8) are believed to be central to the pathogenesis of pulmonary fibrotic disorders. While fibroblasts are critical to normal wound healing, their apoptosis is ultimately necessary to prevent excessive matrix deposition and scarring (9). Fibroblasts from patients with pulmonary fibrosis have been shown to exhibit resistance to apoptosis (10, 11). Thus, identifying mediators that modulate fibroblast survival may not only shed light on disease pathogenesis, but also provide targets for the development of novel therapies that may limit or even reverse the progression of this deadly disease. Although one approach is to interrupt the actions of mediators that promote fibroblast survival, such as transforming growth factor (TGF)-β, another approach is to augment pathways that promote fibroblast apoptosis.

Prostaglandin E_2 (PGE_2) is an antifibrotic lipid mediator derived from the metabolism of arachidonic acid by cyclooxygenase. Human idiopathic pulmonary fibrosis (IPF) has been shown to be characterized by a relative deficiency of PGE_2 production (12–14) and responsiveness (15, 16), and the latter has also been observed in animal models of lung fibrosis (13). Elaborated from lung epithelial cells (17), fibroblasts (14), and macrophages (18, 19), PGE_2 inhibits fibroblast proliferation (18, 19), collagen synthesis (20), migration (21, 22), and differentiation into myofibroblasts (23). However, PGE_2 has also been shown to be a potent antiapoptotic/prosurvival mediator in normal epithelial cells (24, 25), cancer cells (26, 27), T cells (28, 29), and adipocytes (30). Whether PGE_2 is also antiapoptotic in normal lung fibroblasts, or instead promotes apoptosis, which would be consistent with its other antifibrotic actions, is unknown.

The purpose of our study was to delineate the effects of PGE_2 on lung fibroblast survival and the intracellular mechanisms that mediate them. Our results show for the first time that PGE_2 promotes fibroblast apoptosis through activation of multiple apoptotic pathways. This is dependent on signaling through the E prostanoid (EP) 2 and 4 receptors and activation of phosphatase and tensin homologue on chromosome 10 (PTEN)

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with downstream inhibition of protein kinase B/Akt, a known prosurvival signaling mediator. Interestingly, fibroblasts from some patients with IPF are resistant to the proapoptotic effect of PGE_2. These findings reveal another dimension by which PGE_2 may act as an antifibrotic mediator and contrast with the well-recognized role of PGE_2 as a prosurvival mediator in other cell types.

MATERIALS AND METHODS

Reagents

PGE_2, PGD_2, the prostacyclin analog iloprost, the thromboxane A_2 analog U-4419, and the EP2-specific agonist butaprost free acid were purchased from Cayman Chemical (Ann Arbor, MI, USA). The specific caspase 8 inhibitor Z-IETD-FMK, caspase 9 inhibitor Z-LEHD-FMK, and camptothecin (CPT) were purchased from Calbiochem (San Diego, CA, USA). Activating antibody for the Fas (CD95) receptor (FasL/CD95L/APO-1) was obtained from Millipore (Temecula, CA, USA). Cycloheximide (CHX) was obtained from Sigma-Aldrich (St. Louis, MO, USA). The EP3-specific agonist ONO-AE3–248 and the EP4-specific agonist ONO-AE1–329 were generously provided by Ono Pharmaceuticals (Osaka, Japan). Antibodies for poly-ADP-ribose polymerase (PARP), Akt, phospho-Akt, PTEN, and survivin were obtained from Cell Signaling (Danvers, MA, USA); α-tubulin was obtained from Sigma-Aldrich.

Cell culture

Primary normal fetal lung fibroblasts (IMR-90) were obtained from the Coriell Institute for Medical Research (Camden, NJ, USA). Fibroblasts from patients with usual interstitial pneumonia (UIP), the characteristic histological abnormality of IPF, were grown from lung explants derived from biopsy specimens obtained during diagnostic evaluation. Normal adult control fibroblasts were grown from histologically normal margins of tissue resected for lung cancer and from cell lines (CCL201) obtained from American Type Culture Collection (Manassas, VA, USA). All patient-derived cells were isolated as described previously (31). This study was approved by the University of Michigan Institutional Review Board, with all patients providing informed consent. Murine embryonic $PTEN^{-/-}$ and embryonic C57BL/6 wild-type fibroblasts were isolated as described previously (21). All cells were cultured in Dulbecco's modified Eagle medium (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (Hyclone, Logan, UT, USA), and 1% penicillin/streptomycin (Invitrogen). Cells were studied between passages 5–10.

Annexin V-FITC/propidium iodine (PI) staining

Cells were treated for 24 h in serum-free medium under the indicated conditions. Detached cells were collected by centrifugation and combined with adherent cells; cells were then resuspended in Annexin V binding buffer (BD Biosciences, San Diego, CA, USA). Cells were then incubated with 5 μl of Annexin V-FITC (which binds to phosphatidylserine, present only on the outer membrane of apoptotic cells) and 5 μl of PI (which binds to DNA) for 15 min at room temperature. Cells were analyzed and quantitated by flow cytometry.

PARP cleavage

PARP is a target for cleavage by caspases, and increases in cleaved PARP are indicative of apoptotic cells. Both intact and cleaved PARP was detected by immunoblotting. Cells (5×10^5) grown in 6-well plates were treated under the indicated conditions for 24 h in serum-free medium before being collected in lysis buffer (phosphate-buffered saline with 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS). Any nonadherent cells were centrifuged and combined with the adherent cells for preparation of lysates. Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and subjected to immunoblot analysis using anti-PARP antibody (1:1000).

Single-stranded DNA assay

Denaturation of DNA results in single-stranded DNA (ssDNA) that is selectively found in condensed chromatin of apoptotic cells. We measured ssDNA by immunoassay, as described previously (32). Cells (5×10^3) grown in 96-well plates were treated for 24 h in serum-free medium. DNA was then denatured with formamide, and ssDNA was detected by a monoclonal antibody (Chemicon, Billerica, MA, USA). After washing, the bound antibody was detected by ABTS chromophore (Calbiochem) and quantitated on an absorbance plate reader, with values corrected for cell count.

Caspase 3, 8, and 9 assays

Caspase 3 activity was measured in cell lysates using the Caspase-3 Fluorometric Assay Kit (Assay Designs, Ann Arbor, MI, USA), according to manufacturer's protocol. All cells were treated under the indicated condition in serum-free medium for 24 h before being collected for assay. Caspase 8 and 9 activities were measured in lysates using the Caspase 8 and Caspase 9 Fluorometric Assay Kits (R&D Systems, Minneapolis, MN, USA), performed according to manufacturer's protocol. All values were normalized for protein concentration.

PTEN siRNA

IMR-90 cells were transfected using Lipofectamine LTX (Invitrogen) with either 100 nM of PTEN siRNA (Cell Signaling) or nonspecific scrambled siRNA (Cell Signaling). Cells were studied at 48 h after transfection.

Immunoblotting

Proteins from cell lysates were separated by SDS-PAGE and transferred to nitrocellulose membranes for immunoblotting, as described previously (33). Antibodies for Akt, phospho-Akt, PTEN, survivin, and α-tubulin were used at 1:1000; antibody for Fas receptor was used at 1:500. Bound primary antibodies were visualized with appropriate secondary antibody conjugated to horseradish peroxidase and developed with enhanced chemiluminescence reagent (GE Healthcare, Piscataway, NJ, USA). Densitometric analysis was performed using Scion Image (U.S. National Institutes of Health, Bethesda, MD, USA).

Data analysis

Statistical analysis was performed using either ANOVA or Student's t test, as appropriate, using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). A value of $P < 0.05$ was considered statistically significant.

RESULTS

PGE_2 elicits a dose-dependent increase in fibroblast apoptosis

To determine the effects of PGE_2 on lung fibroblast survival, we treated IMR-90 cells for 24 h with increasing doses of PGE_2 (10–1000 nM) and measured cellular apoptosis by annexin V staining, caspase 3 activity, cleavage of PARP, and ssDNA levels (**Fig. 1**). In all assays, PGE_2 elicited a dose-dependent increase in apoptosis (EC_{50}~50 nM), with maximal effects seen at 500–1000 nM. Vehicle control consisting of medium with 0.01% dimethyl sulfoxide (which corresponds to the amount of dimethyl sulfoxide in the 1000 nM PGE_2 treatment condition) elicited no significant increase in apoptosis over that observed in untreated cells. PGE_2 treatment resulted in an increase in annexin V^+/PI^- cells (early apoptosis), but no change in annexin V^+/PI^+ (late apoptosis/necrosis) or annexin V^-/PI^+ cells (necrosis).

We next examined the effects of PGE_2 on fibroblast survival in the presence of another apoptotic stimulus. FasL plus cycloheximide have been shown to induce fibroblast apoptosis, and impaired susceptibility to FasL-induced apoptosis has been observed in cells from patients with pulmonary fibrosis (10, 11). Combined treatment with PGE_2 and FasL/cycloheximide led to more apoptosis than did either alone (**Fig. 2**). This finding shows that PGE_2 augments fibroblast apoptosis in the presence of another known apoptotic stimulus.

PGE_2 induces apoptosis *via* EP2-EP4 receptor signaling

PGE_2 can signal through 4 distinct G-protein-coupled EP receptors (EP1–4). In lung fibroblasts, the $G\alpha_s$-coupled EP2 is the most abundantly expressed (33) and mediates the signaling necessary for inhibition of collagen synthesis, cell proliferation (33), and fibroblast migration (21). To determine which EP receptors are responsible for PGE_2-induced apoptosis, we treated cells with specific EP2, EP3, and EP4 agonists. An increase in apoptosis was observed with agonists for both of the $G\alpha_s$-coupled EP2 and EP4 receptors but not with the $G\alpha_i$-coupled EP3 agonist (**Fig. 3**). Interestingly, among other prostanoids tested, PGD_2, which is produced predominantly by mast cells and signals through the $G\alpha_s$-coupled DP receptor, was also able to elicit apoptosis. A stable analog of thromboxane A_2, which signals through the $G\alpha_i$-coupled TP receptor, did not. Iloprost, a stable analog of prostacyclin, which signals through the $G\alpha_s$-coupled IP receptor, elicited a degree of apoptosis that was slightly greater than untreated control cells. (Supplemental Fig. 1).

Both intrinsic and extrinsic pathways mediate PGE_2-induced apoptosis

The development of apoptosis can proceed through various pathways (34). The intrinsic, or mitochondrial, pathway is activated under conditions of metabolic stress; cytochrome *c* is released from the mitochondria

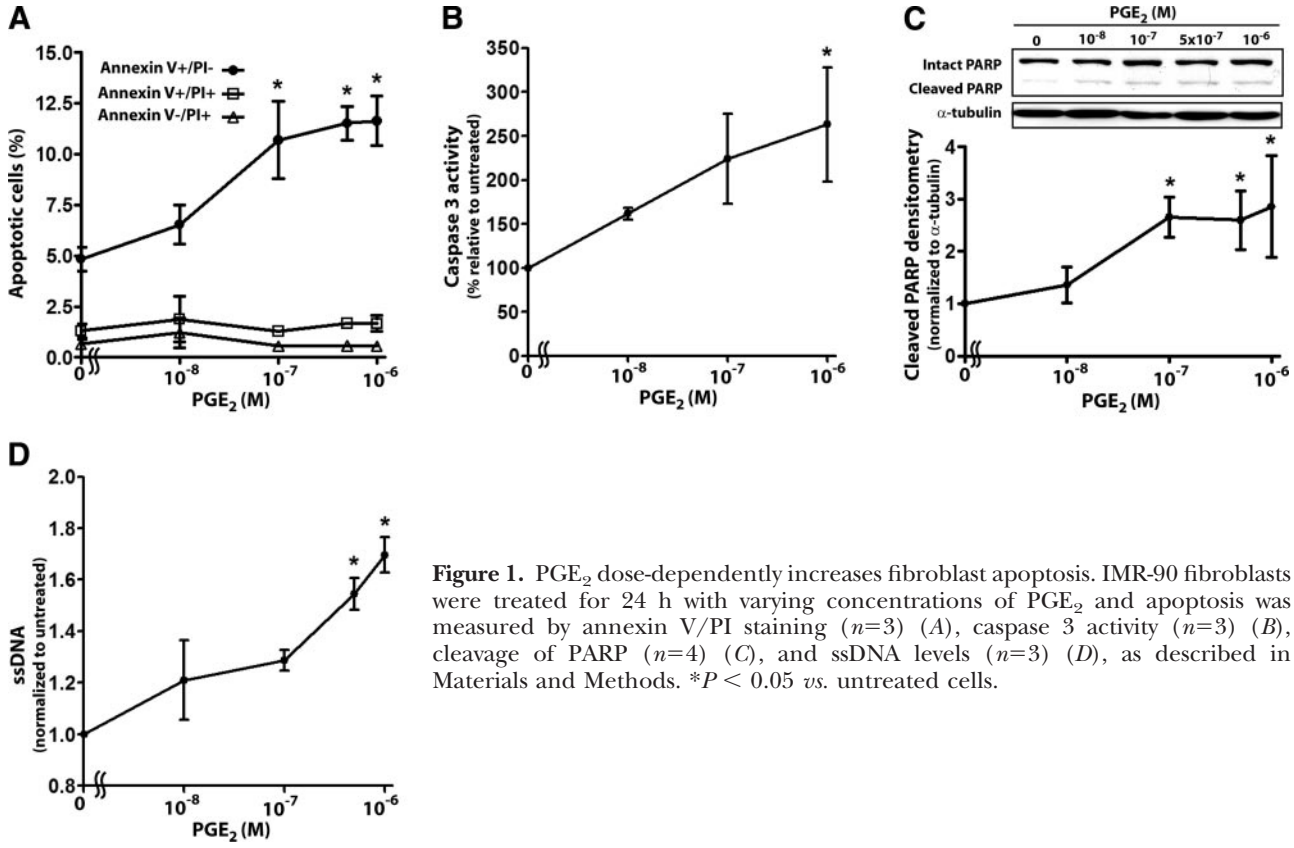

Figure 1. PGE_2 dose-dependently increases fibroblast apoptosis. IMR-90 fibroblasts were treated for 24 h with varying concentrations of PGE_2 and apoptosis was measured by annexin V/PI staining (n=3) (*A*), caspase 3 activity (n=3) (*B*), cleavage of PARP (n=4) (*C*), and ssDNA levels (n=3) (*D*), as described in Materials and Methods. *$P < 0.05$ *vs.* untreated cells.

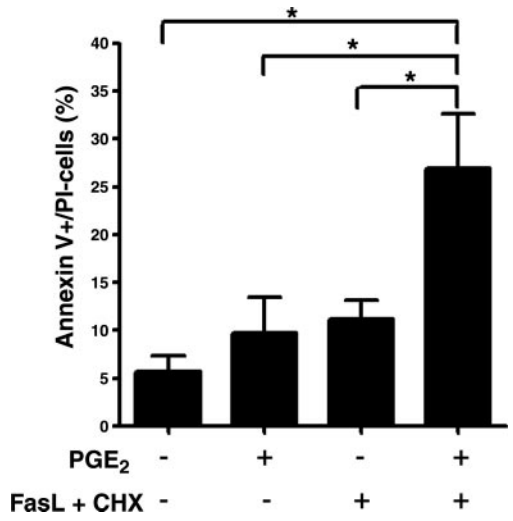

Figure 2. PGE_2 potentiates FasL-induced apoptosis of fibroblasts. IMR-90 fibroblasts were treated in the presence or absence of PGE_2 (0.1 μM) and FasL (100 ng/ml) plus CHX (0.5 μg/ml) for 24 h, and cellular apoptosis was measured by annexin V/PI staining (n=3). Results are expressed as the percentage of annexin V^+/PI^- (early apoptosis) cells. $^*P < 0.05$.

with activation of the apoptosome complex containing caspase 9, which leads to further cleavage of downstream effector caspases 3, 6, and 7 (35). The extrinsic, or death receptor, pathway involves the binding of extracellular signals (*i.e.*, FasL, TNF-α) to death receptors (*i.e.*, Fas), which, in turn, triggers the formation of the death-induced signaling complex, which leads to activation of caspase 8 (36, 37); activated caspase 8 further cleaves downstream effector caspases.

To determine which pathway might be responsible for PGE_2-induced apoptosis, we first measured the activities of both caspases 8 and 9 in cells treated with PGE_2. PGE_2 induced a 25 ± 7.8% increase in caspase 8 activity and a 48 ± 10.9% increase in caspase 9 activity ($P<0.05$ for both). Furthermore, both the caspase 8 inhibitor Z-IETD-FMK and the caspase 9 inhibitor Z-LEHD-FMK (**Fig. 4*A, B***), at concentrations used in other studies (38, 39), independently inhibited PGE_2-induced apoptosis. These findings suggest that PGE_2 requires activation of both caspase 8 and caspase 9 to induce fibroblast apoptosis.

PTEN is required for PGE_2-induced apoptosis

PTEN is a lipid phosphatase that antagonizes the phosphatidylinositol-3-kinase (PI3K) pathway by dephosphorylating phosphatidylinositol 3,4,5-trisphosphate (PIP_3) (40). PIP_3 is a lipid mediator that recruits and activates proteins with pleckstrin homology domains such as Akt, which has been shown to be an important prosurvival mediator (41) that can inhibit both the intrinsic (42) and extrinsic (43) apoptotic pathways. Previous work has shown that PGE_2 activation of PTEN, with subsequent loss of Akt activity, is responsible for PGE_2 inhibition of fibroblast migration (21). We sought to determine whether this same pathway is responsible for PGE_2-mediated apoptosis of fibroblasts.

We first sought to examine the apoptotic effects of PGE_2 in murine fibroblasts from embryonic $PTEN^{-/-}$ mice ($PTEN^{-/-}$ mice do not survive to gestation). As expected, PGE_2 was unable to decrease Akt phosphorylation in $PTEN^{-/-}$ fibroblasts, in contrast to its ability to do so in wild-type embryonic fibroblasts (**Fig. 5*A***). Unlike control wild-type embryonic fibroblasts, fibroblasts from $PTEN^{-/-}$ mice were likewise not susceptible to apoptosis by PGE_2 (Fig. 5*B*). However, these cells were still susceptible to the alternative inducer of apoptosis, the topoisomerase inhibitor CPT. These findings suggest that activation of PTEN and subsequent inhibition of Akt is necessary for PGE_2 to induce apoptosis.

To confirm the importance of PTEN in PGE_2-induced apoptosis, we also silenced PTEN expression in IMR-90 cells using siRNA (**Fig. 6*A***). As with cells from $PTEN^{-/-}$ mice, fibroblasts treated with PTEN siRNA similarly failed to undergo apoptosis in response to PGE_2 but were susceptible to cell death evoked by CPT (Fig. 6*B*). These findings show that PTEN is critical to PGE_2-induced apoptosis in fibroblasts.

PGE_2 decreases survivin expression and increases Fas receptor expression

Akt activates multiple downstream pathways that are responsible for its prosurvival effects, including the induction of multiple antiapoptotic genes (41). These include the inhibitors of apoptosis proteins (IAPs) which directly inhibit caspases. Among the IAPs, sur-

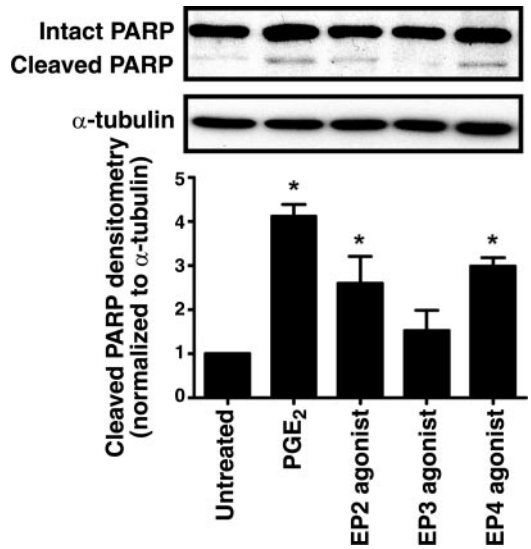

Figure 3. PGE_2 elicits apoptosis *via* EP2/EP4 signaling. IMR-90 cells were treated for 24 h with PGE_2 (500 nM), the EP2-specific agonist butaprost free acid (BFA; 500 nM), the EP3-specific agonist ONO-AE3–248 (100 nM), or the EP4-specific agonist ONO-AE1–329 (100 nM), and apoptosis was measured by cleavage of PARP. Top panel: representative blot. Bottom panel: densitometric analysis of cleaved PARP relative to α-tubulin (n=3). $^*P < 0.05$ *vs.* untreated control.

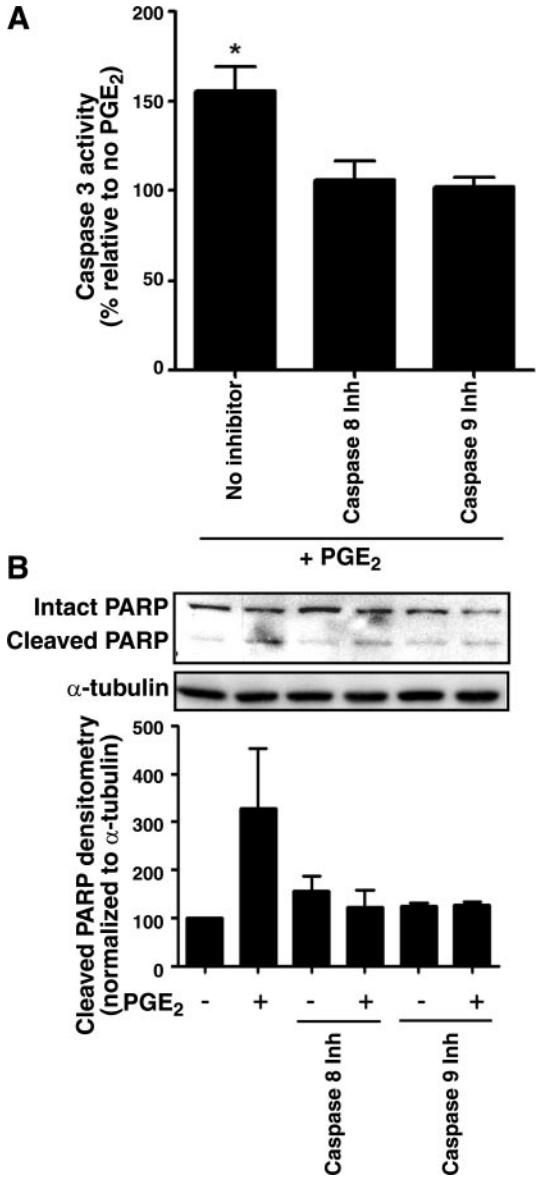

Figure 4. PGE_2 promotes fibroblast apoptosis *via* both caspases 8 and 9. *A*) IMR-90 fibroblasts were treated with PGE_2 (0.5 μM) in the presence or absence of the caspase 8 inhibitor Z-IETD-FMK (25 μM) or the caspase 9 inhibitor Z-LEHD-FMK (25 μM) for 24 h. Caspase 3 activity was measured as described in Materials and Methods; data are expressed as a percentage of activity relative to the no-PGE_2 control (n=4). *$P < 0.05$. *B*) IMR-90 fibroblasts were treated with PGE_2 (0.5 μM), Z-IETD-FMK (25 μM), and/or Z-LEHD-FMK (25 μM) for 24 h. Lysates were immunoblotted for cleaved PARP. Top panel: representative immunoblot. Bottom panel: densitometry of cleaved PARP normalized to α-tubulin (n=3).

vivin has been shown to be important in lung cancer (44) and colorectal cancer cells (45). Cancer cells exhibit increased survivin expression, which directly inhibits caspases (46). It is down-regulated by Akt signaling (47) and has been shown to be up-regulated by PGE_2 in lung cancer cells (48). Because survivin is a target of both Akt and PGE_2 signaling, we examined its expression in fibroblasts treated with PGE_2. Survivin expression was dose-dependently inhibited by PGE_2 (**Fig. 7*A***), which is in contrast to the action of this prostanoid observed in cancer cells (48, 49).

Because Akt has also been shown to protect cells from the extrinsic apoptotic pathway (50) and because we found that PGE_2 potentiates FasL-induced apoptosis, we also examined the effects of PGE_2 on expression of the Fas receptor. PGE_2 dose-dependently increased Fas receptor expression (Fig. 7*B*). Together, these data show that PGE_2 can affect expression of multiple targets that all enhance fibroblast apoptosis.

Variable resistance to PGE_2-induced apoptosis is observed in fibroblasts from patients with UIP

We examined the apoptotic response to PGE_2 of fibroblasts from patients with UIP/IPF. Previous work in our

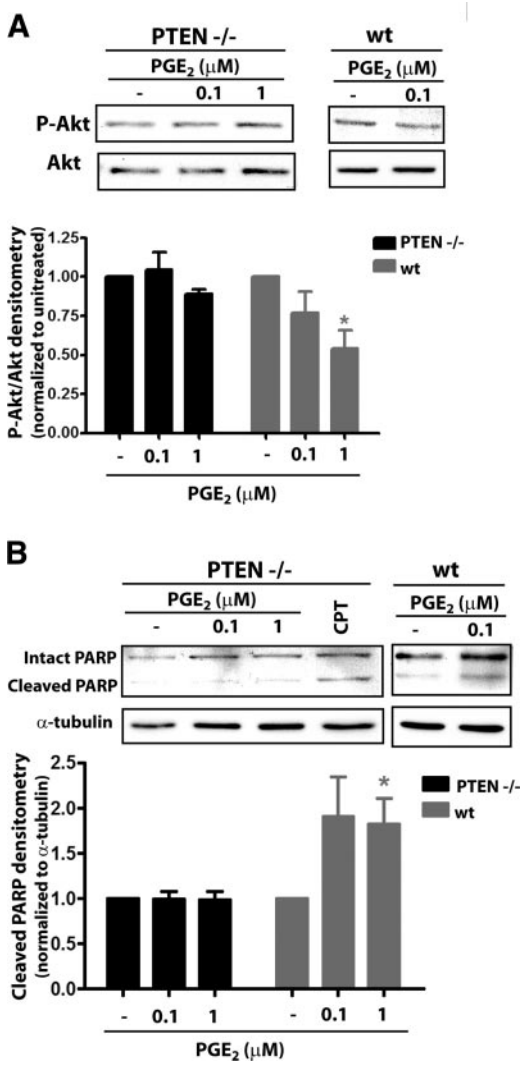

Figure 5. Diminished capacity for PGE_2-induced apoptosis in $PTEN^{-/-}$ fibroblasts. $PTEN^{-/-}$ and wild-type (wt) murine embryonic fibroblasts were treated for 24 h with PGE_2 at the indicated concentrations. Phosphorylated Akt (*A*) and cleaved PARP (*B*) were assayed by immunoblot analysis. Top panels: representative immunoblots. Bottom panels: relative densitometry normalized to untreated controls (wt, n=3; $PTEN^{-/-}$, n=4). *$P < 0.05$.

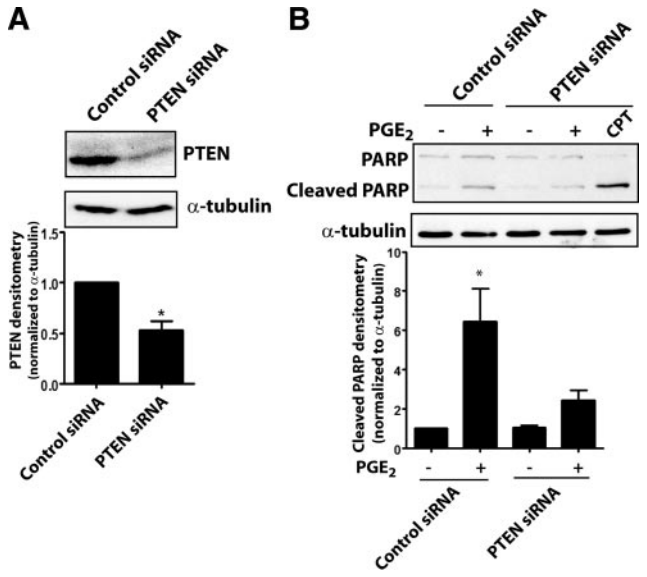

Figure 6. PTEN silencing diminishes PGE_2-induced apoptosis in fibroblasts. *A*) PTEN expression was assayed by immunoblot in cells treated with control or PTEN siRNA for 48 h. Top panel: representative blot. Bottom panel: mean densitometric data (n=4). *$P < 0.05$. *B*) PTEN siRNA- and control siRNA-treated cells were exposed to PGE_2 (0.1 μM) for 24 h, and cleaved PARP was assayed by immunoblot. Top panel: representative blot. Bottom panel: densitometry of cleaved PARP/α-tubulin (n=3). CPT (2 μg/ml) was used as a positive control. *$P < 0.05$ *vs.* no-PGE_2 treatment.

laboratory has shown that in many of these patient-derived cell lines, fibroblasts are resistant to the suppressive effects of PGE_2 on collagen expression and cell proliferation (16). Each of the three nonfibrotic fibroblast lines exhibited dose-dependent apoptosis in response to PGE_2. By comparison, the group of 8 lines of UIP fibroblasts exhibited a relative degree of resistance to PGE_2-elicited apoptosis (**Fig. 8*A***). However, when the responses of individual UIP lines were examined, 3 lines exhibited dose-related apoptosis to PGE_2, while 5 lines were mainly resistant. This variable response among different UIP fibroblast lines is consistent with the variable inhibitory effects of PGE_2 on collagen expression and cell proliferation, which we observed previously (16).

Previous studies have also shown that UIP fibroblasts are resistant to FasL-induced apoptosis (10, 11), and data in Fig. 8*B* support this conclusion. In control cells, PGE_2 potentiated the proapoptotic effects of FasL/cycloheximide; although this appeared to be the case in the UIP cell lines as well, the degree of potentiation was less than observed in normal cell lines (Fig. 8*B*). A summary of PGE_2-mediated apoptotic signaling is shown in **Fig. 9**.

DISCUSSION

PGE_2 has been shown to be antiapoptotic in multiple cell types, including normal epithelial cells (24, 25), cancer cells (26, 27), T cells (28, 29), and adipocytes (30). Despite the well-known contribution of fibroblast survival to the accumulation of this key mesenchymal cell in tissue fibrosis, there are few studies that have addressed the effects of this prostanoid on fibroblast survival. Our studies demonstrate for the first time that in contrast to its predominant antiapoptotic effect in other cell types, PGE_2 promotes apoptosis of lung fibroblasts. These findings provide a novel mechanism by which PGE_2 may mediate antifibrotic effects.

The pathobiology of pulmonary fibrosis is characterized by an increase in epithelial cell apoptosis (2–4) and a decrease in fibroblast apoptosis (5–8). The importance of TGF-β as a profibrotic mediator derives in part from its ability to promote each of these opposing effects—a phenomenon that has been termed the "apoptosis paradox" (7, 51). That PGE_2 acts as an antiapoptotic signal in epithelial cells (24, 25) but as a proapoptotic signal in fibroblasts therefore signifies another such apoptosis paradox and emphasizes the pleiotropic antifibrotic potential of PGE_2. The fact that impaired synthesis of PGE_2 in pulmonary fibrosis (12–14) would be expected to promote fibroblast survival may explain the persistent fibrogenesis observed in this disease.

Our studies employing both RNA silencing and gene

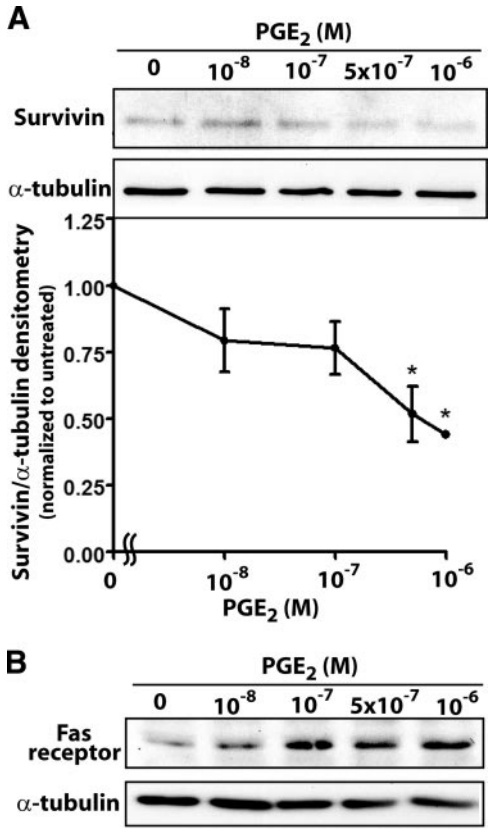

Figure 7. PGE_2 modulates fibroblast expression of survivin and Fas receptor. IMR-90 fibroblasts were treated for 24 h with the indicated concentrations of PGE_2, and survivin expression (n=3) (*A*) and Fas receptor expression (n=3) (*B*) were determined by immunoblot analysis. *$P < 0.05$ *vs.* no-PGE_2 treatment.

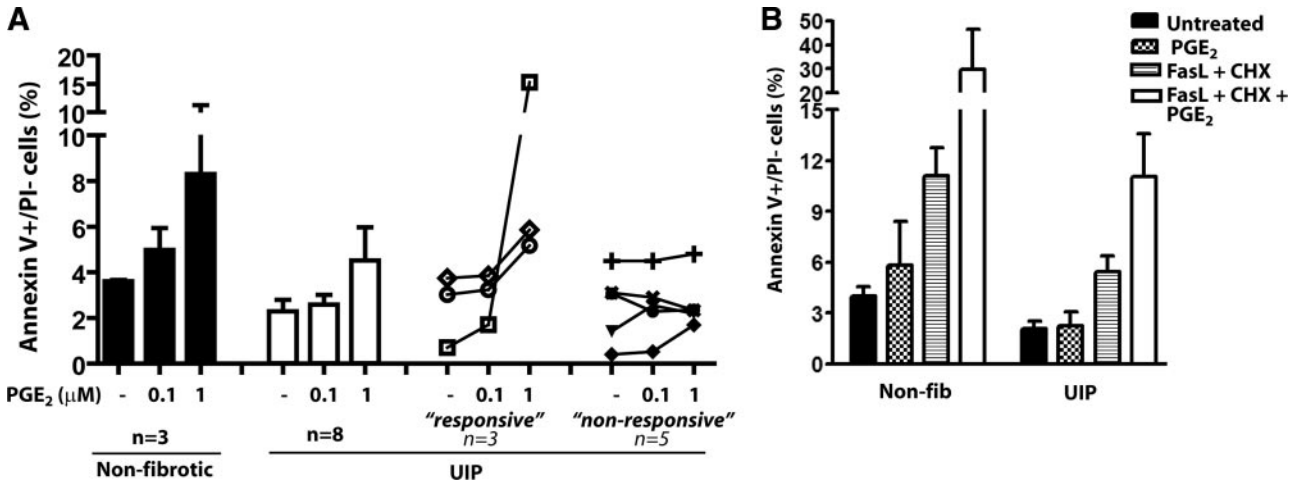

Figure 8. PGE_2-induced apoptosis in adult control and UIP fibroblasts. *A*) Fibroblasts from the lungs of patients with UIP (n=8, white bars) and nonfibrotic controls (n=3, black bars) were treated with PGE_2 for 24 h, and apoptosis was measured by annexin V/PI staining, with results expressed as the percentage of annexin V^+/PI^- (early apoptosis) cells. Responses of individual UIP lines are displayed at right, segregated within responsive and unresponsive groups. *B*) UIP (n=3) and nonfibrotic control (n=4) fibroblasts were treated with PGE_2 (0.1 μM) ± FasL (100 ng/ml) + CHX (0.5 μg/ml) for 24 h. Cells were stained for annexin V and PI; percentage of annexin V^+/PI^- cells is shown.

deletion indicate that PGE_2-induced fibroblast apoptosis is dependent on PTEN activation; this results in down-regulation of Akt, a molecule that has been shown to be important in prosurvival signaling (41). The contrasting actions of PGE_2 in fibroblasts *vs.* epithelial cells may be explained by differences in PTEN/Akt signaling, since we have shown that PGE_2 activates PTEN and inhibits Akt (21) in the former, while it activates Akt in epithelial (24) and cancer cells (52). Although the measured increases in caspase 8 and caspase 9 activities were modest, both appear to participate in PGE_2-induced apoptosis on the basis of data using selective inhibitors. The importance of both the intrinsic (as reflected by the role for caspase 9) and extrinsic (as reflected by the role for caspase 8) apoptosis pathways is not surprising, given their recognized capacity for crosstalk (53).

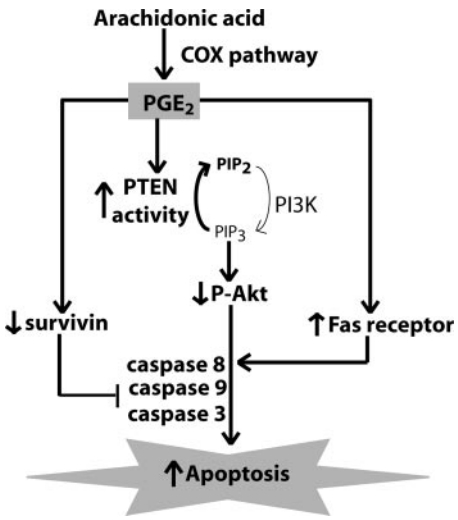

Figure 9. Schema of PGE_2-induced apoptosis in lung fibroblasts. PGE_2, synthesized by the cyclooxygenase (COX) pathway, induces apoptosis by increasing PTEN activity and decreasing Akt activity. PGE_2 also decreases survivin and increases Fas expression in lung fibroblasts. PGE_2-induced apoptosis is mediated by ligation of the EP2 and EP4 receptors; relative contributions of EP2 and EP4 signaling to the 3 apoptosis pathways remain to be determined.

We furthermore show for the first time that PGE_2 inhibits expression of survivin. This once again contrasts with findings in other cell types, in which PGE_2 was shown to increase survivin expression in cancer cells (48, 49), thereby contributing to its role in promoting oncogenesis. We also found that PGE_2 increases expression of Fas. These findings contrast with reports of PGE_2 decreasing FasL expression in T cells, thereby preventing T-cell activation-induced cell death (29). This contrasting action on the Fas/FasL pathway further illustrates that actions of PGE_2 are cell-type dependent.

Previous studies have shown that a chemical inhibitor of Akt, LY294002, does not increase baseline fibroblast apoptosis (32), suggesting that increased PTEN activity/decreased Akt activity by PGE_2 may be necessary, but not sufficient, for PGE_2-induced apoptosis. Rather, this may require the contributions of additional proapoptotic pathways, including increased Fas expression and decreased survivin expression. Indeed, increased Fas expression could explain our finding that PGE_2 potentiates apoptosis elicited by FasL plus cycloheximide. The possible interrelationship among increased Fas expression, decreased survivin expression, and PTEN/Akt signaling requires further investigation, and the relative contributions of EP2 and EP4 receptor signaling to these three mechanisms remain to be determined. However, the observation that PGE_2 modulates multiple determinants of apoptosis in lung fibroblasts may explain its ability to activate both intrinsic and

extrinsic pathways, as well as its potential to influence apoptosis in diverse biological contexts.

PGE_2 synthesized by neighboring epithelial cells or fibroblasts themselves may act as a paracrine or autocrine brake to limit excessive fibroblast accumulation under normal homeostatic conditions. Since PGE_2 enhanced FasL-induced apoptosis even in UIP fibroblasts, which were previously shown to be resistant to FasL signaling (10, 11), it may play an important role in sensitizing cells to apoptosis. Thus, the modest magnitude of PGE_2-induced apoptosis does not diminish its biological importance, and the actions of PGE_2 may help potentiate other apoptosis-targeted therapies.

A previous study has shown that PGE_2 protects against fibroblast apoptosis in the presence of cigarette smoke extract (54). These experiments were performed in cells treated for only 6 h, and the effects of PGE_2 alone were not tested. Our findings instead examined the effects of PGE_2 both alone and with the addition of FasL, employing later time points that may be more relevant to the kinetics of apoptosis. Nevertheless, the seemingly disparate findings between the two experimental models may also point to the pleiotropic actions of PGE_2 in the presence of differing apoptotic signals.

Studies in UIP fibroblasts demonstrated that many, but not all, cell lines exhibited resistance to the pro-apoptotic effects of PGE_2. This mirrored our previous findings in which some but not all UIP fibroblast lines exhibited resistance to PGE_2-mediated inhibition of collagen synthesis and cell proliferation (14). Multiple mechanisms (diminished EP2 expression, diminished protein kinase A expression, and activity) accounted for resistance to PGE_2 inhibition of collagen synthesis and cell proliferation (14). Given that PGE_2-induced apoptosis proceeds *via* EP2/EP4 receptor activation, it is likely that apoptosis resistance in UIP cells reflects these same mechanisms responsible for resistance to PGE_2 inhibition of collagen synthesis and cell proliferation. Interestingly, decreased PTEN expression has also been described in fibroblasts from some UIP patients (55), possibly also contributing to their resistance to apoptosis.

Although our studies address PGE_2-mediated fibroblast apoptosis *in vitro*, the *in vivo* relevance of this phenomenon remains to be determined. However, it is of interest that the cyclooxygenase inhibitor indomethacin was shown to worsen bleomycin-induced fibrosis in mice (56). As PGE_2 is the major cyclooxygenase-derived prostanoid produced by both alveolar epithelial cells (57) and fibroblasts (57), it has the capability to elicit fibroblast apoptosis in both a paracrine and autocrine fashion. Although we found that PGD_2 likewise possesses this capability, its relevance in the setting of parenchymal fibrogenesis is less apparent.

CONCLUSIONS

As opposed to normal and malignant epithelial cells, for which PGE_2 is antiapoptotic, we show that PGE_2 promotes apoptosis of lung fibroblasts, and that this involves enhanced PTEN signaling and decreased Akt activation. PGE_2 also decreased survivin and increased Fas receptor expression. A resistance to PGE_2-induced apoptosis was observed in some UIP cells, which could be overcome with the combination of FasL and cycloheximide. Although PGE_2 has previously been demonstrated to inhibit the activation of diverse fibrogenic functions in lung fibroblasts, its ability to promote apoptosis offers a potential opportunity not merely to prevent progression of pulmonary fibrosis but to intervene in and potentially reverse established disease. FJ

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